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Growth rate and identification of *Fusarium* spp. assosiated with *Aquillaria* spp. from Nunukan regency, North Kalimantan

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A B S T R A C T

Fusarium spp is one of the fungi associated with *Aquillaria* spp. in producing agar wood as non-timber forest products. Agarwood formation is triggered by fungal infection or abiotic stress. Based on these facts, the formation process of agar wood in the stem is considered as chemically plant defense mechanisms against unfavorable condition. The aim of this study was to identify several *Fusarium* isolates associated with agar wood trees based on their morphological and molecular characters. *Fusarium* isolates were collected from *Aquillaria* spp. trees in four Districts of Nunukan, North Kalimantan, with different heights, ranging from 0 to 2000 m above sea level: South Nunukan (0–100 masl), Lumbis (100–500 masl), Krayan Induk (1000–1500 masl), and South Krayan (1000–2000 masl). *Fusarium* isolates were cultivated on Potato Dextrose Agar (PDA) and observed their colony colour (surface and reverse), size, shape and separate of macroconidia, conidiophore, as well as the presence of chlamidospore and sporodochia. Furthermore, they were cultivated on 7 different media to test their growth rate and production of conidia. For molecular characterization a part of large subunit rRNA gene of *Fusarium* isolates were amplified by PCR, cloned and sequenced. The results showed that entirely 11 isolates belong to Genus *Fusarium*. They have different ability to grow on 7 kinds of growth medium. One isolate from Krayan Induk (KR1) showed a restricted growth on all kinds of growth medium. Molecular analyze using PCR followed by sequencing, revealed that isolates from South Nunukan (NKS), Krayan Induk (KR2), South Krayan (KRS1, KRS2 and KRS5) were identified as *F. solani*, isolates from Lumbis (LM1) as *Fusarium* sp, and LM2 isolate as *F. fujikuroi*. KR1 isolate has homology sequence with *F. oxysporum* whereas KRS4 identified as *F. ambrosium.*

Introduction

Agar wood (*Aquilaria malaccensis*) belong to a high economy value of non timber forest products (NTFPs) and since hundred years ago has been used as medicine and for diverse industrial purposes. It has many usages as raw material of perfume, air freshener, incense, cosmetics and traditional medicine (Sudrajat, 2003). Agar wood is

also used in rituals and religious ceremonies and beliefs devotional objects such as beads and rosary (Barden *et al.,* 2000).

Tropical natural forest exploitation and uncontrolled hunting of agar wood has resulted in aloe extinction. Therefore in 1995 the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) has incorporated *Aquillaria malaccensis,* in Appendix II and in 2004 the entire species of *Aquillaria* spp., and *Gyrinops* spp., included in Appendix II, with the goal to protect the species from extinction (Semiadi *et al.,* 2009). As a commodity that provides a very large income in Indonesia, the sustainable utilization should be maintained (Sulistyo, 2010).

Most aloes are found in the injured tree aloes, either naturally by abiotic factors such as wind, rain or lightning, as well as biotic by microorganisms. Abiotic factors are factors that are difficult to imitate because it cannot be the basis of the production processes in aloe industry (Mucharromah, 2010). Abiotic factor is also not systemic that can spread to other parts of healthy plant (Santoso *et al.,* 2010). The formation of agar wood by biotic factors may be caused by microorganisms. The mechanism of the formation of agar wood by microorganisms can be spread to any other part of the plant because the microorganisms perform all the activities required for life in the plant tissues (Santoso *et al.,* 2010).

Microorganisms that commonly found infecting agar wood trees and producing aloes are fungal groups i.e. *Fusarium* spp., *Phytium* sp., *Lasiodiplodia* sp., *Libertela* sp., *Trichoderma* sp., *Syctalidiums* sp. *Thielaviopsis* sp. but the most dominant species were *Fusarium* (Sumarna, 2002). *F. solani,* (Mart) Appel and Wollenw., *F. lateritium* Ness., *F. tricinctum* (Corda) Sacc., and *F. moniliformae* Sheldon., were reported can infect agar wood trees (Budi *et al.,* 2010).

A wide range of media are used for growing fungi. Most mycologists develop preferences for certain types of media that are routinely grown. Type of media will affect colony morphology and color, formation of specific structures, and may affect if the fungus will even grow in culture. For example, some fungi lack the necessary enzymes to utilize different carbon sources. All fungi require several specific elements for growth and reproduction. The requirements for growth are generally less stringent than for sporulation, so it is often necessary to try several types of media when attempting to identify a fungus in culture. Most fungi thrive on Potato Dextrose Agar (PDA), but this can be too rich for many fungi, so that excessive mycelial growth is obtained at the expense of sporulation. Most of the fungi isolated from soil, or from substrates in the soil, i.e., plant debris, grow well on Corn Meal Agar (CMA), a relatively weak medium compared to PDA. Similarly, wood-inhabiting fungi and dematiaceous (dark pigmented) fungi often sporulate better on CMA or Oat Agar, both of which have less easily digestible carbohydrate than PDA. Cellulose-destroying fungi and spoilage fungi retain their ability to produce cellulase when grown on a weak medium such as Water Agar (WA) or Potato Carrot Agar (PCA) with a piece of sterile filter paper, wheat straw or lupin stem placed on the agar surface. The introduction of pieces of tissue, such as filter paper, wheat straw, rice, grains, leaves or dung, often produces good sporulation dependent on the organism grown.

Morphological identification of plant pathogenic fungi is the first and the most difficult step in the identification process. This is especially true for *Fusarium* species. Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage. However, for species that cannot be reliably identified in this way, additional analysis such as DNA sequencing and species-specific PCR assays must be conducted.

Based on these reasons, it is necessary to study about the diversity of *Fusarium* isolates that associated in *Aquillaria* spp. trees and their ability to grow on different growth medium. The information will be very important for the production of agar wood, so that the status of scarcity or extinction of agar wood plants become a sustainable product.

Material and Methods

Isolation of fungal isolates

All isolates were obtained from surface sterilized stem of *Aquillaria* spp. Isolation and purification of isolates was revealed on Potato Dextrose Agar (PDA). Purified isolates were then identified based on their morphological characterization according to identification key of Nelson *et al.* (1983), and Leslie and Brett (2006). Determination of colony color refers to the Chart of Rayner (Rayner, 1970).

Growth rate on different mediums

Measurement of the fungal growth rate of was conducted on Malt Pepton Agar (MPA), Malt Ekstrak Agar (MEA), Water Agar (WA), Coconut Water Media, Potato Extract, Modified PDA and extract of agar wood leaf. All of isolates were incubated at room temperature with adequate light exposure. Each treatment was replicated three times. Furthermore, isolates were also tested for their ability to produce conidia on liquid medium. Conidia density was calculated 7 days after incubation using the formula of Susilo (1993).

Molecular identification

DNA extraction was performed according to Moeller (1992). For amplification of DNA, a large ribosomal subunit 28S rRNA Nuclear gene (LROR: ACCCGCTGA ACTTAAGC, LR5: TCCTGAGGGAA ACTTCG) was used as primer. PCR analysis performed with 50 µL total reaction containing 5 µL DNA, 2 µL 10 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 6 µL $MgCl_2$, 1 µL of each primer, 1.25 units of Taq enzyme polymerase, and 10 µL 10X PCR buffer. The PCR program consisted of pre-denaturation at 95ºC, for 15 minutes, followed by 35 cycles consisting of denaturation at 94ºC for 60 seconds, annealing at 50ºC for 60 seconds, and extension at 72ºC for 60 seconds followed by Final Extension for 10 minutes at 72ºC.

PCR products were electrophoresed in a 2% agarose gel in 0.5X TAE solution (Tris Acetic Acid EDTA). A total of 8µl PCR product mixed with 2µl of loading dye, loaded in the gel electrophoresis wells, and done at a voltage of 100 volts for 60 minutes. Documentation was conducted on UVP High Performance Ultraviolet Transluminator (Biorad).

The presence of bands was analyzed by comparing the results of the test sample with a 100 bp marker on the left and right sides. PCR products were then sequenced to determine the base sequence of each isolate and compared with *Fusarium* species collected in Genbank (National Center for Biotechnology Information/NCBI).

Results and Discussion

Morphological identification

Based on morphological characters, entirely fungal isolates from four districts with different altitude in Nunukan regency belong to the Genus *Fusarium*. The isolates have varied color, from white to dark purple, straight to curved macroconidia with 3–7 septate and one or two celled microconidia (Table 1 and Figure 1).

Growth rate of isolates on several different media

Cultivation of isolates on different mediums showed, that each isolate have different ability to grow on medium with different nutrition content. KR1 isolate growth very slow on all mediums and reach only 3.30 cm after 7 days incubation. KRS2 and KRS3 isolates grow faster on all kinds of medium and reach maximal diameter at 5 days after incubation (data not shown). In comparison with other medium, Agar wood Leaf Agar medium, gave the lowest growth on 11 isolates tested (Table 2.). In other hand, production of conidia on different liquid media revealed that use coconut water medium can trigger the production of conidia. Similar result was also observed on growth medium supplemented with potato extract (Table 3.).

Molecular identification

Molecular characterization was revealed by using of 28S RNA primer pairs (LROR and LR5). Electrophoresis of PCR product from 11 DNA isolates generated one band in size of 900 bp (Figure 2). There were difference in thickness of DNA band among isolates, but no band was detected on isolate No. 2 (KR1). It was suggested that no enough DNA content in this isolate. In comparison with other isolates, the growth rate of this isolate on solid and liquid culture was very limited. Based on this result, KR1 isolate was not further analyzed to sequence.

Sequencing result of all isolates was compared with data base of *Fusarium* species found in Genbank (National Center for Biotechnology Information/NCBI). Percentage of similarity of each isolate with *Fusarium* species was presented in Table 4.

Species of the genus *Fusarium* are well-researched in many fields, such as ecology, plant pathology, medical-mycology and toxicology (Pitt and Hocking, 2009). One problem commonly encountered by researchers interested in *Fusarium* species is the probable taxonomic system and the identification method of this Genus. *Fusarium* colony is growing fast with pale or bright color. Some isolates have cottony aerial mycelium and their thallus varied from whitish to yellow, brownish, pink, reddish or purple in color. *Fusarium* species produce hyaline, two- to several celled macroconidia and 1–2 celled, hyaline, pyriform, fusiform to ovoid, straight or curved microconidia (Leslie and Brett, 2006).

Fungi grown on light conditions continuously to form aerial mycelium relatively large, aerial mycelium is formed with a relatively large abundance phototropi mechanism to the presence of light (Irawati, 2004). However, in this study all isolates tested were treated the same light, so that the presence of an abundance of aerial mycelium due to the character of each isolate. Colony colour of isolates was also different. The diversity found in colony color associated with pigment contained by the cell wall of hyphae. Pigmented fungi are not generally colorless or hyaline.

The growth of the fungus sporulation can be influenced by the presence of light. Hawker (1971) reported several species are cultured in a Petri dish shows macroconidia production zone in response to light. Light can induce the length and number of septa macroconidia on several species of *Fusarium* spp. Seifert (1996) states that macroconidia be observed from colonies grown on SNA medium (Synthetic Nutrient Agar), CLA (Carnation Leaf Agar), or BLA (Banana Leaf Agar).

Amplification of target DNA by PCR followed by sequencing is potentially more sensitive and faster than microbiological techniques. PCR is more sensitive for the detection of *Fusarium* species selectively on plants infected agar wood. Isolation and identification of fungi that can infect *Aquilaria* spp and induce aloes formation have been found several types *Fusarium*. The results of the study revealed that the molecular identification of 10 isolates were studied, 5 isolates belong to *F. solani* (Mart) Appel & Wollenweber Emend, Snyder & Hansen (NKS, KR2, KRS1, KRS2, and KRS5. LM1 isolate belong to *Fusarium* sp., whereas LM 2 identified as *F. fujikuroi* Nirenberg. LM3 isolate was identified as *F. oxysporum* Schlechtendahl Emend, Snyder & Hansen and KRS 4 as *F. ambrosium* (Wollenweber). One isolate (KR1) was not further analyzing cause of it slightly mycelium production in liquid culture for DNA extraction process. There are four species of *Fusarium* spp were identified, namely *F. solani*, *Fusarium* sp, *F. fujikuroi, F. oxysporum* and *F. ambrosium*. Among the four species, *F. solani* is the most commonly found species. *F. solani* is an important pathogen on *Aquilaria* spp. Booth (1971) and Nelson *et al.* (1983) reported that *F. solani, F. oxysporum, F. fujikuroi,* and *F. ambrosium* are cosmopolitan species, because some of the species in this group is a saprophyte. Several studies that have been done previously reported the presence of *F. solani* on agar wood plants. Sidiyasa and Suharti (1987) states that different kinds of fungi such as *Diplodia* sp., *Pythium* sp. and *F. solani* have an important role in the formation of agar wood resin. Furthermore Umboh *et al.* (2000) using the fungi *F. oxyforum, F solani, Scytalidium sp., Libertella sp.,* and *Trichoderma* sp. to induce the aloe formation in *Aquilaria malaccensis* sapwood and *A. crassna* Pierre ex LaConte.

In general, species are recognized on the basis of the morphological species concept, the biological species concept, the phylogenetic species concept or a combination of these (Taylor *et al.* 2000). The traditional taxonomic system for fungi has been proposed based on the mainly morphological species concept, including the genus *Fusarium*. The taxonomy of this genus has been debated for many years (Booth, 1971). Recently, many researchers have applied molecular phylogenetic analysis to examine the taxonomy of *Fusarium* species, and have proposed new taxonomic systems based on the phylogenetic species concept. These molecular approaches achieved some positive results in the phylogenetic problems which could not be elucidated with morphological markers.

Tentative identification using morphological characters grouped the isolates in *Fusarium* Genera. All 11 *Fusarium* isolate have different growth rate either in solid medium or in liquid medium. Based on the results of the molecular identification, of 11 isolates collected from four districts in Nunukan with different sea level altitude, 5 isolates were belong to *Fusarium solani*, two isolates refers to *Fusarium oxysporum*, and each one isolate identified as *Fusarium* sp, *F. fujikuroi*, and *F. ambrosium*.

Table.1 Morphological characters of *Fusarium* isolates

Isolate Code	Origin	Macro- and microconidia	Surface Colony Color on Malt Peptone Agar
NKS	South Nunukan	Present	White, Purple
KR 1	Krayan Induk 1	Present	White
KR 2	Krayan Induk 2	Present	Light Purple
LM 1	Lumbis 1	Present	Light Purple
LM 2	Lumbis 2	Present	White, Purple
LM 3	Lumbis 3	Present	Dark Purple
KRS 1	South Krayan 1	Present	White, Purple
KRS 2	South Krayan 2	Present	White
KRS 3	South Krayan 3	Present	White
KRS 4	South Krayan 4	Present	White
KRS 5	South Krayan 5	Present	White, Light Purple

Table.2 Growth rate of 11 *Fusarium* isolates of 7 different mediums, 7 days post incubation (dpi)

No	Code of Isolat	Growth Diameter (cm) 7 days post incubation (dpi)						
		Agar wood Leaf	Modif. PDA	Potato Extract	CWA	WA	MEA	MPA
1	NKS	6.50	7.05	7.40	7.50	6.97	7.73	6.67
2	KR1	2.83	3.30	2.17	2.93	2.30	2.60	2.33
3	KR2	8.37	6.23	8.67	8.20	6.90	6.83	5.87
4	LM1	6.33	4.83	7.47	7.67	5.97	7.63	4.57
5	LM2	6.33	5.43	6.60	7.27	6.10	7.60	5.37
6	LM3	6.00	5.30	7.43	7.27	6.50	7.00	5.40
7	KRS1	7.47	9.00	9.00	9.00	6.47	7.97	6.07
8	KRS2	6.80	8.37	9.00	8.93	6.37	8.27	9.00
9	KRS3	8.00	5.57	7.67	7.27	6.10	7.53	9.00
10	KRS4	4.47	7.97	7.53	8.67	6.80	7.93	7.53
11	KRS5	4.67	6.53	7.47	7.53	6.53	7.43	6.67

Table.3 Spore concentration of 11 *Fusarium* isolates on different liquid medium

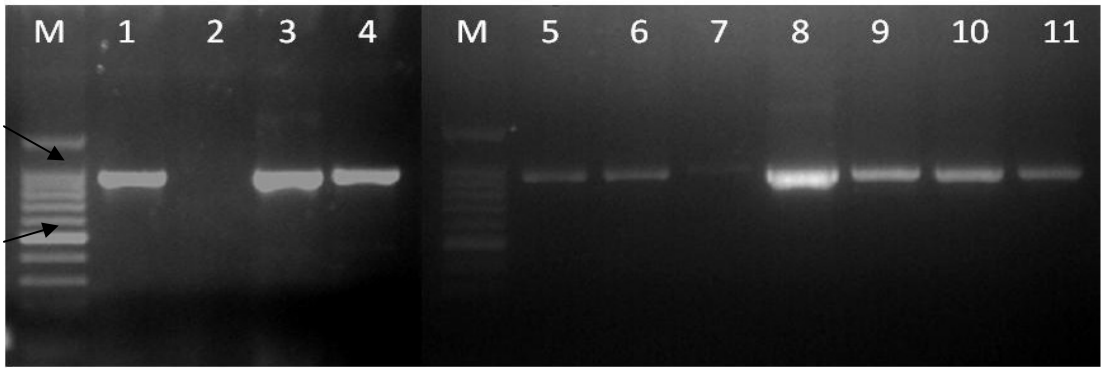
No.	Isolate	Coconut water	Potato Extract	Modified PDA	MPA	MEA	CDA
1.	NKS	$246x10^6$	$55x10^6$	$65x10^6$	$6x10^6$	$14x10^6$	$2x10^6$
2.	KR1	$78x10^6$	$10x10^6$	$30x10^6$	2x106	$4x10^6$	$1x10^6$
3.	KR2	$103x10^6$	$70x10^6$	$57x10^6$	$6x10^6$	$14x10^6$	$1x10^6$
4.	LM1	$85x10^6$	$42x10^6$	$43x10^6$	$9x10^6$	$15x10^6$	$5x10^6$
5.	LM2	$89x10^6$	$86x10^6$	$56x10^6$	$8x10^6$	$14x10^6$	$2x10^6$
6.	LM3	$92x10^6$	$53x10^6$	$25x10^6$	$7x10^6$	$19x10^6$	$2x10^6$
7.	KRS1	$40x10^6$	$100x10^6$	$17x10^6$	$9x10^6$	$10.3x10^6$	$1.2x10^6$
8.	KRS2	$93x10^6$	$78.3x10^6$	$37x10^6$	$46x10^6$	$14x10^6$	$1.3x10^6$
9.	KRS3	$234x10^6$	$68x10^6$	$50x10^6$	$46x10^6$	$26x10^6$	$2x10^6$
10.	KRS4	$90x10^6$	$81x10^6$	$51x10^6$	$28.3x10^6$	$15x10^6$	$3.3x10^6$
11.	KRS5	$335.3x10^6$	$50x10^6$	$37.3x10^6$	$16x10^6$	$14x10^6$	$1.3x10^6$

Table.4 Identification of 11 *Fusarium* isolates based on nucleotide sequence homologies

No.	Isolate Code	Reference Strains	Nucleotide sequence homology (%)
1.	NKS	*F. solani* strain CBS102256	92%
2.	KR 1	-	-
3.	KR 2	*F. solani* strain CBS102256	99%
4.	LM 1	*Fusarium* sp CBG_I7CCH	74%
5.	LM 2	*Fusarium oxysporum* strain KAML01	96%
6	LM 3	*F. fujikuroi*	91%
7.	KRS 1	*Fusarium solani* strain CBS102256	87%
8.	KRS 2	*Fusarium solani* strain CBS102256	98%
9.	KRS 3	*Fusarium oxysporum* strain KAML01	96%
10.	KRS 4	*F. ambrosium* strain SMH1999	88%
11.	KRS 5	*F. solani* strain CBS102256	87%

Figure.1 Growth rate and surface colony color of 11 *Fusarium* isolates on Malt Peptone Agar (MPA) 7 dpi (days post incubation)

Figure.2 Electrophoresis of DNA *Fusarium* after PCR using primer pairs of LROR-LR5. 2 % Agarose and visualization with Sybrgreen



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